AVIAN DISEASES 53:169–174, 2009

Protection Against Fowl Cholera Conferred by Vaccination with Recombinant *Pasteurella multocida* Filamentous Hemagglutinin Peptides

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Received 24 September 2008; Accepted and published ahead of print 11 November 2008

SUMMARY. Three gene fragments, derived from *Pasteurella multocida* strain P-1059 (serotype A:3), encoding approximately the 5′ one-third of *fhaB*2 were overexpressed individually in *Escherichia coli*. The recombinant peptides were purified, pooled, and administered to turkey poults to evaluate immunity. The results showed that turkeys immunized twice with the recombinant peptides were significantly protected against intranasal challenge with *P. multocida* strain P-1059. Vaccination elicited antibody responses, based on Western blotting, that were reactive with a wild-type P-1059 cellular product approximately 170 kDa in size and multiple high molecular weight products in culture supernatant. These antibodies did not react with cell or supernatant blots of a P-1059 *fhaB2* isogenic mutant. *Pasteurella multocida fhaB*2 genes of a bovine strain (A:3) and an avian strain (F:3) are highly conserved as is the portion of P-1059 *fhaB*2 examined here (>99% identities). These findings suggest that broad cross-protection against this heterogeneous pathogen may be achievable through immunization with specific recombinant FHAB2 peptides.

RESUMEN. Protección contra la cólera aviar conferida por la vacunación con péptidos recombinantes de la hemoaglutinina filamentosa de *Pasteurella multocida*.

Tres fragmentos de genes derivados de la cepa P-1059 de *Pasteurella multocida* (serotipo A:3), que codificaban aproximadamente el tercio orientado al extremo 5′ del gen *fhaB*2 (hemoaglutinina filamentosa), fueron expresados individuamente en *Escherichia coli*. Los péptidos recombinantes fueron purificados, combinados y administrados a pavitas para evaluar la inmunidad. Los resultados mostraron que los pavos inmunizados con los péptidos recombinantes fueron protegidos de manera significativa contra el desafío intranasal con *P. multocida* cepa P-1059. La vacunación estimuló respuestas de anticuerpos, que con base en la inmunoelectrotransferencia, mostraron reacción con un producto celular de una cepa P-1059 de campo, el producto mostró un tamaño de aproximadamente 170 kDa. También se observó reacción con múltiples productos de alto peso molecular del sobrenadante del cultivo. Estos anticuerpos no reaccionaron con las células ni con los sobrenadantes de una mutante isogénica P-1059 del gen *fhaB*2. Los genes *fhaB*2 de *Pasteurella multocida* de una cepa bovina (A:3) y de una cepa aviaria (F:3) están altamente conservados como la porción del gen *fhaB*2 de la cepa P-1059 examinada en este estudio (con identidades mayores de 99%). Estos hallazgos sugieren que una protección cruzada amplia contra este patógeno heterogéneo puede lograrse a través de la inmunización con péptidos recombinantes FHAB2 específicos.

Key words: *Pasteurella multocida*, filamentous hemagglutinin, recombinant vaccine

Abbreviations: bp = base pairs; CFU = colony forming unit; CPF = cross-protection factor; FC = fowl cholera; FHA = filamentous hemagglutinin; SDS = sodium dodecyl sulfate; TBS = Tris buffered saline; WT = wild type

Pasteurella multocida is the causative agent of fowl cholera (FC), a highly contagious disease that affects all species of birds, and the turkey industry is the most severely affected sector of commercial importance. Predominately, *P. multocida* serotypes A:1, A:3, and A:3,4 are responsible for most FC outbreaks in poultry flocks. It is thought that *P. multocida* principally enters host tissues through mucus membranes of the pharynx or upper air passage, resulting in septicemia accompanied by high morbidity and mortality. Current commercial vaccines against FC include bacterins and live attenuated strains. The adjuvanted bacterins generally provide limited protective immunity and do not control disease by different serotypes (6). The commercial attenuated live vaccines are recognized as providing broad protection across serotypes, but their precise genetic lesions are uncharacterized, and outbreaks associated with such vaccines have occurred (2).

Bacterial surface proteins are successfully used to impart protective immunity in a range of infections. A specific outer membrane antigen of *P. multocida*, referred to as cross-protection factor (CPF), isolated from *in vivo* grown bacteria was reported to elicit heterologous serotype protection against FC (14,15). In additional protection studies, the identity of the CPF was not characterized beyond that of being a 39 kD membrane protein (13). Recently *P. multocida* PlpE protein was postulated as being the CPF protein, and recombinant PlpE was shown to protect both mice and chickens against challenge (21). In another recent study vaccination with recombinant OmpH protein protected mice against *P. multocida* challenge (9).

Pathogen attachment functions are important for establishment of mucosal infections, and mediating these processes are adhesins. Often these pathogens employ multiple attachment factors for colonization of their respective hosts. One approach to the control of microbial infections is to identify specific bacterial factors involved in adherence and/or colonization and apply this knowledge to devise targeted therapeutic interventions to subvert these crucial steps in disease pathogenesis. For example, the filamentous hemagglutinin (FHA) protein of *Bordetella pertussis* plays a critical role in this organism's adherence to host cells (10), and *B. pertussis* FHA is now included as a key component in acellular vaccines against whooping cough (16). Later it was determined that immunization with an N-terminal 80 kD fragment of *B. pertussis* FHA protected mice against *B. pertussis* challenge (1). In *Bordetella bronchiseptica*, FHA is critical for lower respiratory tract colonization (4), immunomodulation, and the ability to overcome innate immunity (8). With *P. multocida*, it

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Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′–3′)	Targets
Peptide 1 For	aagaattcGTTCCTGTGGCAGAATG	*fhaB*2 bp 52–68
Peptide 1 Rev	aaaggatccCTTTGCTACGATTTTGGC	*fhaB*2 bp 1341–1324
Peptide 2 For	aagaattcGTAGCAAAGGGTGCC	*fhaB*2 bp 1333–1347
Peptide 2 Rev	aaaggatccATGATTAATTAACTGCGGTTTTG	*fhaB*2 bp 2376–2351
Peptide 3 For	aagaattcAAAGGAACATTTAATGCGGAAGC	*fhaB*2 bp 2389–2411
Peptide 3 Rev	aaaggatccACGATCATCACGACTTATACC	*fhaB*2 bp 3252–3232
KMT1T7	ATCCCGTATTTACCCAGTGG	*P. multocida* specific
KMT1SP6	GCTGAAAACGAACTCGCCAC	*P. multocida* specific

was demonstrated that a filamentous hemagglutinin (*fhaB*2) mutant is highly attenuated in turkeys (18). The reduced pathogenicity of the FHA mutant is hypothesized to be related to decreased capacity to colonize the respiratory epithelium or to an impaired ability to invade tissues. Thus we hypothesize that an effective antibody response to FHAB2 that interferes with either of the mechanistic roles proposed for this protein could prevent disease. Here we have undertaken a study to evaluate the utility of recombinant *P. multocida* FHAB2 peptides as a subunit vaccine against FC.

MATERIALS AND METHODS

Bacterial strains. *P. multocida* strain P-1059 (A:3) was obtained from the collection of Drs. R. Rimler, R. E. Briggs, and F. M. Tatum maintained at the National Animal Disease Center, Ames, IA. Wild-type *P. multocida* P-1059 and a *P. multocida* P-1059 *fhaB*2 mutant used in this study were cultured on dextrose starch agar (Difco, Detroit, MI) containing 5% sheep blood, and cells were harvested into Columbia broth (Difco) containing 10% glycerol to produce stock cultures stored at −80 C. Before use in this study, the wild-type P-1059 challenge strain was passed through a turkey that developed peracute FC following intravenous challenge. Pure *P. multocida* culture was obtained from liver of the infected bird, and sample tissue was stored at −80 C. The challenge inoculum was generated by swabbing the liver sample onto a blood agar plate and incubating at 37 C overnight. The pure *P. multocida* growth was amplified in Columbia broth to an optical density of 0.4 at 600 nm and diluted in Earle's balanced salt solution without phenol red (Life Technologies, Rockville, MD) to obtain the intended challenge dose. The exact colony-forming units (CFUs) in the challenge solution were determined by colony plate counts of serial dilutions.

Recombinant DNA techniques. The *fhaB*2 specific primer pairs were designed by sequence analysis of the *fhaB*2 gene obtained from *P. multocida* strain P-1059. The primer pair sequences depicted in capital letters annealed to specific nucleotides of *fhaB*2, while the lowercase nucleotides at the 5′ termini contained *Eco*R1 (forward primers) and *Bam*HI (reverse primers) recognition sites (Table 1). The restriction enzyme recognition sites were included to facilitate the subsequent cloning of the PCR products. All primers described in this work were custom synthesized with an oligonuleotide synthesizer (Applied Biosystems Inc., Integrated DNA Technologies Inc. Coralville, IA). The *fhaB*2 fragments were amplified using whole cells of stain P-1059 as template and the PCR primer pairs listed in Table 1 added to an EasyStart PCR "Mix-in-a-Tube" (Molecular BioProducts, San Diego, CA). Reaction conditions were 30 cycles, each consisting of the following: 30 sec melting at 95 C, 30 sec hybridization at the appropriate temperature for the particular primer pair, and 90 sec extensions at 72 C. The reaction products were purified with QIAquick spin columns (Qiagen Inc., Valencia, CA), and the DNA products were subjected to *Eco*RI and *Bam*HI digestion. Following phenol chloroform extraction and ethanol precipitation, the DNA fragments were ligated into like digested pGemex-1 (Promega Corp. Madison, WI). The ligation mixtures were transformed into *E. coli* DE-3 (Promega) cells and spread onto Columbia blood agar media plates supplemented with 50 μg/ml ampicillin. The plates were then incubated overnight at 37 C, and individual colonies, transformed with recombinant plasmid, were amplified in broth. Plasmid DNA was purified from broth cultures, and the inserts were assessed for in-frame placement within pGemex-1 by sequence analysis. Selected clones were then transferred to 25 ml LB broth (10 g bacto-tryptone, 5 g yeast, 10 g NaCl, 1 liter H_2O) supplemented with 50 μg/ml ampicillin and grown at 37 C. To induce recombinant protein expression, 0.5 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to the growth medium when cell density reached an A_{600} of 0.7. Four hours after IPTG induction, cells were harvested by centrifugation. In the appropriate *E. coli* host strain, sequences cloned into p-Gemex are expressed as a chimeric protein fused to the gene 10 protein of T7 virus. Purification of the recombinant peptides was accomplished using B-Per Reagent (Pierce, Rockford, IL) according to the manufacturer's directions. All the recombinant products generated in this study were contained primarily in insoluble bacterial inclusion bodies. The purified recombinant peptides were resuspended in phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). Purity, quality, and quantitative estimation of the recombinant proteins were assessed by comparing the extracted preparations to bovine serum albumin standards run on a 4%–15% sodium dodecyl sulfate (SDS) PAGE after Coomassie blue staining. An additional quantitative estimation of the recombinant samples was done using the "Bradford" protein assay (BioRad, Hercules, CA). Vaccine was prepared by combining the recombinant peptides to obtain a concentration of 1 mg/ml in 0.9% saline followed by mixing with adjuvant (Titer Max Gold, CyRx Corp., Norcross, GA) 50:50 (volume:volume) according to the manufacturer's directions. A commercial breed of 6-wk-old mixed sex turkeys (Willmar Poultry Company, Willmar MN) were injected intramuscularly with the pooled recombinant FHAB2 fragments (50 μg total) mixed with adjuvant in a volume of 200 μl. Also at this time, control birds were injected with 50 μg purified T7 gene 10 protein mixed with adjuvant as above. Two weeks later booster immunizations identical to the first was given. Sera were collected from each bird at the time of the first vaccination and again 3 wk later.

Vaccination and challenge studies in turkeys. One week after the second boost with the recombinant FHAB2 peptides or the T7 gene 10 protein control peptide the turkeys were challenged intranasally with 1.1×10^7 CFUs of *P. multocida* strain P-1059 in a volume of 0.2 ml. After challenge the birds were observed three times daily at 08:00, 16:00, and 21:00 for signs of disease over the duration of the experiment. Birds showing clinical signs of FC, such as ruffled feathers, ataxia, or dehydration, were euthanatized by intravenously injection of sodium pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA). Tracheal samples were collected by direct swabbing. Liver and spleen specimens were aseptically collected by puncturing the surfaces with a sterile scalpel and rolling the swab in the opening until saturated. The swabs were rolled onto a quarter of Columbia blood agar base plates, and then three consecutive quadrants were streaked with a sterile loop for colony isolation. Representative colonies were subjected to PCR analysis using *P. multocida* specific primers, KMT17T and KMT1SP6 (19), to confirm *P. multocida* growth on the plates. Seven days after infection the remaining birds were euthanatized by intravenous injections, and sample specimens were processed and examined as described above. This animal experiment was approved by the Animal

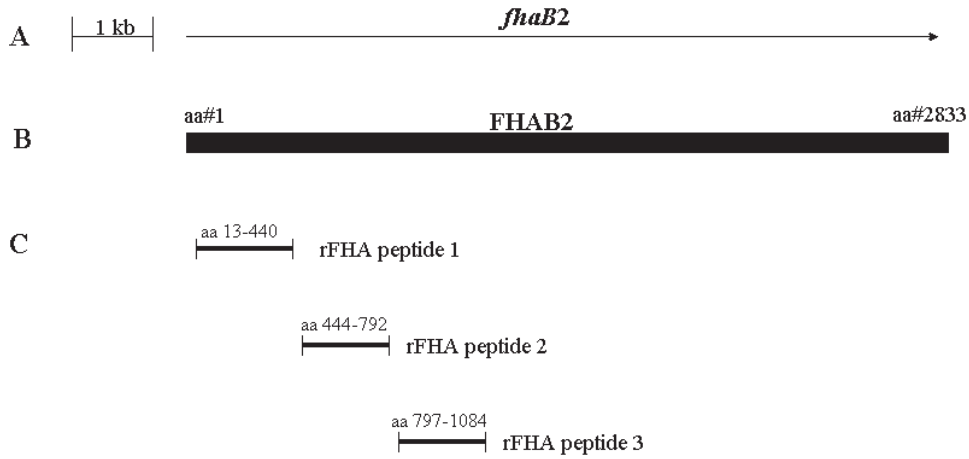

Fig. 1. (A) Schematic representation of the *fhaB*2 gene of *P. multocida* strain P-1059. The arrow indicates the length and direction of the gene. (B) Representation of the 311 kD protein encoded by *fhaB*2. (C) *fhaB*2 fragments cloned into pGemex-1, which generated the rFHAB2 peptides1, 2, and 3, respectively.

Care and Use Committee at the National Animal Disease Center, Ames, IA.

The vaccine trial conducted in this study recorded the survival rates and the mean times to death. The control and vaccinates were compared by chi-squared tests.

Western blotting. Wild-type *P. multocida* P-1059 and a *P. multocida* P-1059 *fhaB*2 mutant were grown in 5 ml Columbia broth to midlog phase and treated with 250 units of hyaluronidase type 1-S (Sigma Chemical Co., St. Louis, MO) for 10 min to remove capsule, which aided cell harvesting. The cells were pelleted by centrifugation at 5000 × *g* for 15 min, and growth media was concentrated 10-fold using Centriprep 30 concentrators (Amicon Inc., Beverly, MA). Fifteen microliters of concentrated culture media or 50 µl midlog culture cells were solubilized in 2× Laemmli buffer (50 mM Tris [pH 6.8], 100 nM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) and subjected to SDS PAGE on a 4%–15% polyacrylamide gel. Running

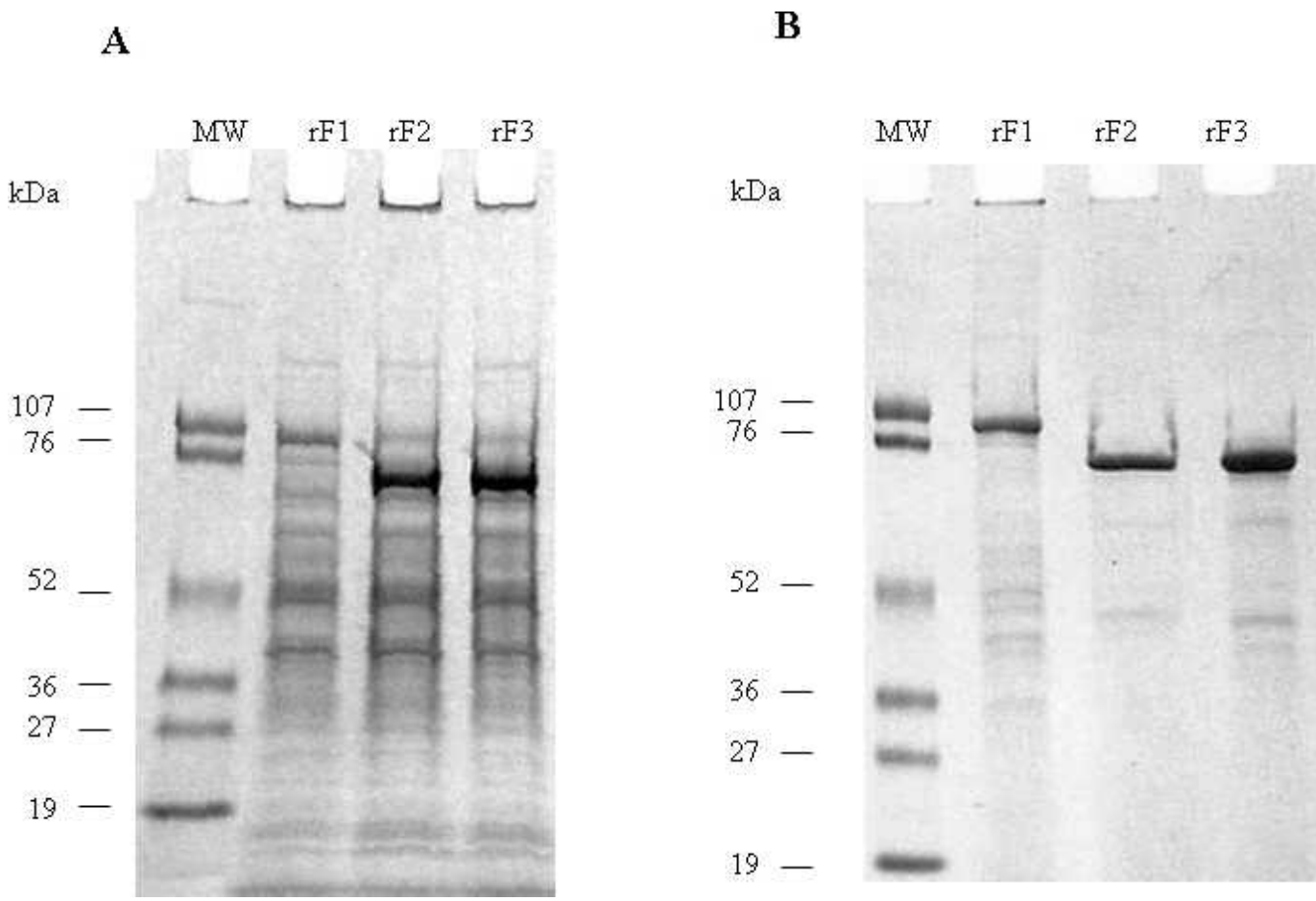

Fig. 2. Expression and purification of rFHAB2 peptides in *E. coli* DE-3 cells. (A) Coomassie blue-stained SDS-PAGE of whole cell lysates expressing the rFHAB2 peptides. (B) Purified samples of the rFHAB2 peptides used as the vaccine.

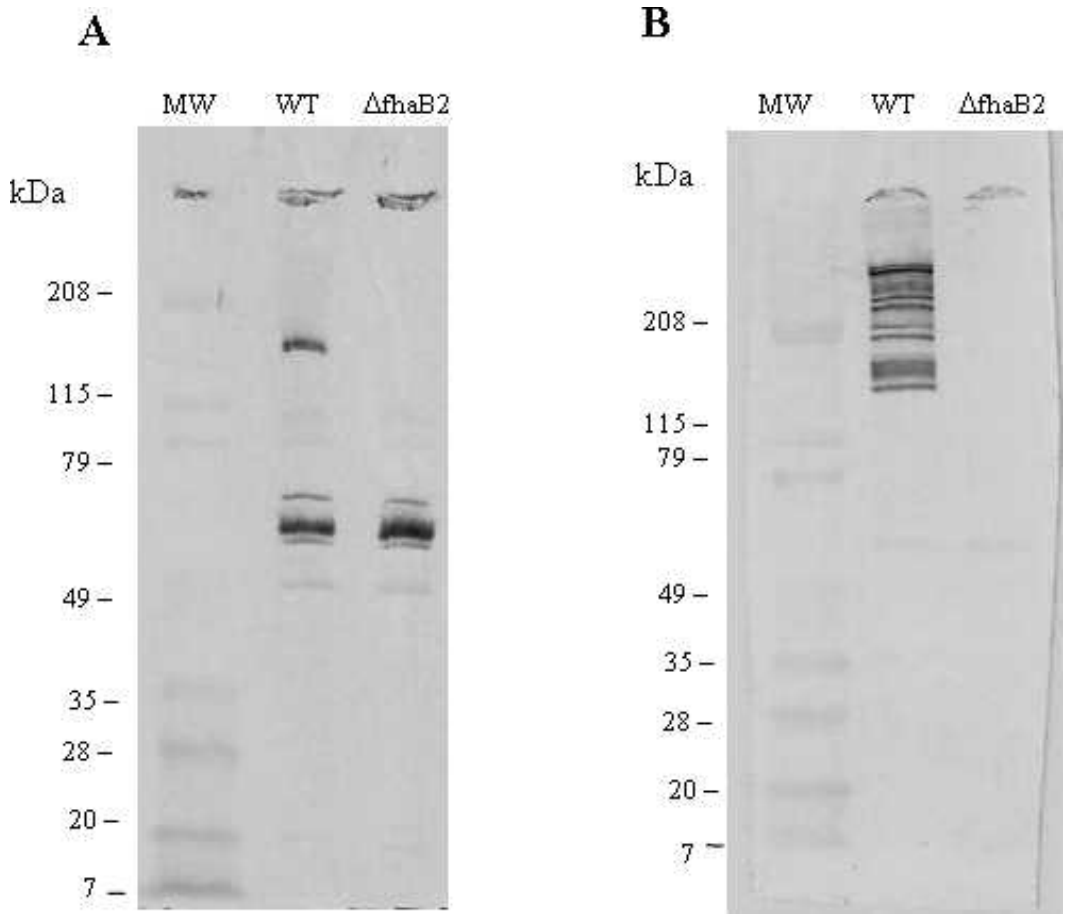

Fig. 3. (A) Western blot analysis of pooled sera from vaccinated turkeys, after immunization and before challenge, reacting to wild-type (WT) or FHA mutant (Δ*fhaB2*) whole cell lysates. (B) Western blot analysis using the same pooled sera as in Fig. 2A reacting to concentrated supernatants of WT or FHA mutant (Δ*fhaB2*) cultures.

buffer consisted of 196 mM glycine, 0.1% SDS, and 50 mM Tris-HCl pH 8.3. On completion of electrophoresis, the separated proteins were either stained with coomassie blue or electrophoretically transferred to BA-S nitrocellulose filters (Schleicher and Schuell, Keene, NH). The nitrocellulose filters were hybridized overnight at 4 C with pooled turkey sera diluted 1:1000 in Tris buffered saline (TBS; 0.05 M Tris; 0.138 M NaCl; 0.0027 M KCl, pH 8.0) blocking buffer containing 5% Carnation instant milk. After washing three times in TBS for 10 min each wash, filters were hybridized for 2 hr at 25 C with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co.) diluted 1:2000 in TBS containing 5% Carnation instant milk. The filters were washed as before and developed in alkaline phosphatase developing buffer (100 mM Tris, pH 9.5; 5 mM $MgCl_2$) containing NBT and BCIP (Sigma).

RESULTS

Expression and purification of recombinant FHAB2 peptides. The three fragments, encoding approximately the initial 1100 amino acids of P-1059 (A:3) *fhaB2* (Fig. 1), were amplified by PCR using the primers indicated in Table 1. The *fhaB2* gene fragments, cloned into p-Gemex1, were expressed as fusion proteins joined to a 260 amino acid N-terminal leader of phage T7 gene 10. The calculated molecular masses of the chimeric recombinant FHAB2 peptides 1, 2, and 3 were 84, 74, and 72 kD, respectively (Fig. 2A). Western blot analysis using goat anti-T7 gene 10 antibody (Bethyl Laboratories, Montgomery, TX) was done (data not shown) to confirm that the expressed recombinant peptides were of the predicted masses. Each of the recombinant peptides, contained primarily in inclusion bodies within *E. coli*, was purified and combined for use as a vaccine candidate against FC (Fig. 2B).

Immunization and challenge studies in turkeys. A commercial breed of 6-wk-old turkeys were injected intramuscularly with 50 μg of purified recombinant FHAB2 peptides in saline with adjuvant. Also at this time 17 control turkeys were treated identically with an inoculum consisting of the T7 gene10 peptide mixed in adjuvant. Two weeks later each bird was treated as before. One week later all turkeys were challenged intranasally with 1.1×10^7 CFUs of low passage wild-type *P. multocida* strain P-1059 resuspended in Earle's balanced salt solution.

All control birds in this study died of acute FC with an average survival time of 49 hr. Pure cultures of *P. multocida* were obtained from all trachea, liver, and spleen specimens sampled from this group. PCR analysis of selected colonies using *P. multocida* specific primers (19) confirmed that the recovered bacterial growths were *P. multocida* serotype A. In contrast, 14 of the 17 birds vaccinated with the recombinant FHAB2 peptides survived intranasal challenge with *P. multocida* P-1059 ($P < 0.001$). All surviving birds exhibited no clinical signs over the duration of this study. After 7 days the remaining vaccinated birds were euthanatized, and tissue samples were obtained. No liver or spleen samples from the vaccinated survivors were culture positive, and only one tracheal sample from this group was positive for *P. multocida* as determined by culturing and PCR analysis. High levels of *P. multocida* were recovered from liver, spleen, and trachea samples taken from the three vaccinates

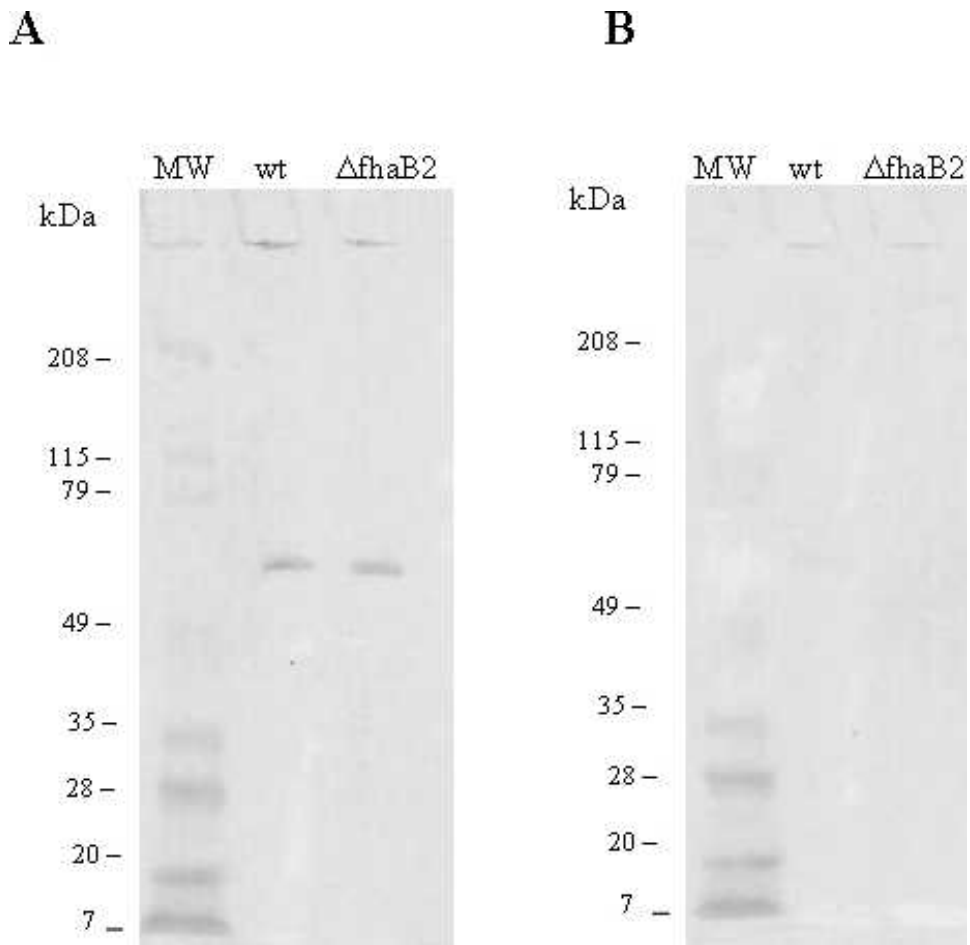

Fig. 4. (A) Western blot analysis of preimmune pooled turkey sera reacting to identical loadings of wild-type (WT) or FHA (Δ*fhaB2*) mutant whole cell lysates as shown in Fig. 2A. (B) Western blot analysis using preimmune pooled turkey sera reacting to identical loadings of concentrated supernatants of WT or FHA mutant (Δ*fhaB*2) cultures as shown in Fig. 2B.

that died following challenge. The average time until death for the vaccinated birds was 105 hr, which was significantly longer than that of controls ($P < 0.05$).

Immune response to the recombinant FHAB2 peptides. Western blot analysis of pooled sera taken prior to bacterial challenge showed that turkeys immunized with the purified recombinant FHAB2 peptides developed antibody titers against a large protein of an approximate mass of 170 kDa present in whole cells (Fig. 3A) and multiple reactive high molecular weight bands present in the concentrated culture media (Fig. 3B). In contrast, blots of cell and culture supernatant of a *P. multocida fhaB2* P-1059 isogenic mutant (Fig. 3A, B) were devoid of reactive products contained in the wild-type parent samples. Pooled prevaccinate turkey sera did not react with the *P. multocida* cellular or supernatant products recognized by the vaccinate sera (Fig. 4B). Both the preimmune and vaccinate sera contained antibodies recognizing an unidentified moiety approximately 65 kD in mass.

DISCUSSION

Filamentous hemagglutinin is a large β-helical protein produced by several *Bordetella* species (10), and this highly immunogenic surface associated and secreted adhesin is a primary component of an acellular pertussis vaccine (16). *Pasteurella multocida* FHAB2 is an important virulence determinate that plays a role in the colonization or cellular invasion of turkeys (18). Here we investigated the efficacy of recombinant FHAB2 subunits as a vaccine to protect turkeys against respiratory challenge by *P. multocida*.

Turkeys immunized with three purified recombinant FHAB2 peptides spanning approximately the first 1050 amino acids of the protein were protected against intranasal challenge with *P. multocida* strain P-1059. At this time we are uncertain as to the relative importance to disease resistance that was provided by the individual FHAB2 peptides examined here. Only the recombinant FHAB2 peptide 1 was determined to possesses homology with a range of filamentous hemagglutinins and hemolysins domains from other bacteria including *Bordetella pertussis* (3), *Escherichia coli* O157 (12), *Pseudomonas aeruginosa* (17), and *Haemophilus ducreyi* (20). This shared domain is associated with carbohydrate-dependent hemagglutination activity.

Western blotting with vaccinate sera demonstrated that a cellular protein with a mass of approximately 170 kDa was present in wild-type *P. multocida* strain P-1059 cellular extract but absent in an *fhaB*2 isogenic mutant (Fig. 3A). The open reading frame of a *P. multocida fhaB*2 gene possesses the capacity to code for a 311-kD protein (11). The discrepancy between the size of the FHAB2 product detected in cells and the predicted product suggests that the protein may undergo posttranslational processing. Such cleavage is reported for *Bordetella pertussis* FHA, as the *fha* gene encodes a predicted product of 367 kD, but the identified mature protein is approximately 220 kD in mass (7). This difference is attributed to

removal of the C-terminal portion of *B. pertussis* FHA. Blotting also demonstrated that wild-type *P. multocida* but not an *fhaB*2 mutant efficiently secreted reactive proteins into the culture supernatant that were recognized by antibodies produced by turkeys injected with the recombinant FHAB2 peptides (Fig. 3B). The multiple sizes of the reactive secreted proteins were unexpected because many of them were larger than the mature FHAB2 protein found in whole cell extracts. Possibly the secreted FHAB2 forms aggregates with itself or with other secreted products to generate the extracellular assemblages observed in the culture media.

With the goal of improving broad vaccine efficacy against *P. multocida* disease, ongoing research is directed toward exploring in more detail the functional importance of individual FHAB2 domains in infection and in the induction of protective immunity. Interestingly *P. multocida fhaB*2 genes of a bovine strain (A:3) (5) and an avian strain (F:3) (11) are highly conserved (>99% identity), as is the region of P-1059 *fhaB*2 used to generate the peptides examined in this study. Thus, based on the results reported here, broad cross protection against various strains of this heterogeneous organism may be achievable through immunization with specific recombinant FHAB2 peptides.

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ACKNOWLEDGMENT

This work was supported by the Biotechnology and Research Development Corporation, Peoria, IL.